Reference: *Biol. Bull.* **169:** 602–614. (December, 1985)

SPERM TRANSFER AND STORAGE IN THE BROODING BIVALVE *MYSELLA TUMIDA*

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ABSTRACT

Mysella tumida has a seasonal reproductive cycle at Patricia Bay, Vancouver Island, Canada. The onset of brooding in the population is preceded by a period of exogonadal sperm storage, which persists for 1–4 months. Sperm storage is achieved by the mass attachment of spermatozoa to the abfrontal unciliated surface of gill filaments in the ascending lamellae, by means of fine microvilli which radiate from the acrosomal end of the sperm heads and interdigitate with the gill epithelial cell microvilli. Eggs are spawned into the gill chamber and are fertilized by the stored spermatozoa. Sperm transfer between individuals involves the production, release, and uptake of spermatophores. Spermatophores are released from and gain re-entry to the suprabranchial chamber through the exhalent opening. This method of sperm transfer and storage results in a high fertilization efficiency; *e.g.*, 99.9% of 39,660 eggs spawned by 50 individuals examined were fertilized. Available data indicate that *M. tumida* normally outcrosses, but the possibility of facultative selfing is not excluded.

INTRODUCTION

The majority of bivalve mollusc species are gonochoric broadcast spawners which undergo external fertilization (Sastry, 1979). The smaller members of many bivalve families, however, exhibit some form of brood protection (Sellmer, 1967; Sastry, 1979), a trait typically associated with hermaphroditism (Strathmann *et al.*, 1984). All members of the superfamily Galeommatacea investigated to date, brood their embryos in the suprabranchial chamber (Sellmer, 1967; Sastry, 1979), with the exception of *Entovalva mirabilis* and *Montacuta percompressa* which also utilize the general mantle cavity as a brood chamber (Voeltzkow, 1891; Chanley and Chanley, 1970). Fertilization occurs within the brood chamber, although the method by which the gametes are brought together to achieve fertilization has not been demonstrated for most galeommatacean species.

Oldfield (1964) and Sellmer (1967) speculate that in outcrossing species of brooding bivalves, spermatozoa are released into the surrounding water by one individual and are entrained by the inhalent current of a recipient animal into the brood chamber where they fertilize the eggs. This may explain how fertilization occurs in mantle cavity brooders, such as *Montacuta percompressa* and *Entovalva mirabilis,* but leaves unresolved the question of how sperm of an outcrossing ctenidial brooder gain access to the eggs. Galeommatacean gills are of the eulamellibranchiate type, which have been shown to filter out particles <4 μm in diameter from the water entering the suprabranchial chamber (Mohlenberg *et al.*, 1978). Sperm placed in the inhalent current of the ctenidial brooder *Mysella bidentata* did not enter the suprabranchial chamber and were frequently passed to the animal's mouth and ingested (Ockelmann and Muus, 1978).

Received 21 February 1985; accepted 26 September 1985.

A number of brooding bivalves exhibit some form of sperm storage. In *Montacuta substriata, M. ferruginosa, Pythinella cuneata,* and *Entovalva perrieri*, sperm masses enclosed in membranous envelopes have been observed in the suprabranchial chambers of adults (Oldfield, 1964; Gage, 1968; Ó Foighil, 1985a). Morton describes the occurrence of sperm morulae in the gill chamber of *Pseudopythina subsinuata* (1972) and *Gaimardia finlayi* (1979). An alternate method of sperm storage which involves the en masse attachment of sperm cells to specialized regions of the gill suspensory membranes and gill lamellae occurs in *Xylophaga dorsalis* (Purchon, 1941), *Mysella bidentata* (Deroux, 1961; Ockelmann and Muus, 1978) and *Montacutona compacta* (Morton, 1980). These sperm storing regions have been interpreted as seminal receptacles (Purchon, 1941; Ockelmann and Muus, 1978; Morton, 1980) and the stored sperm are characteristically orientated with their heads pointed towards the receptacle epithelium. The method of sperm-epithelium attachment is unknown.

Species of *Mysella* are hermaphroditic and brood embryos in the suprabranchial chamber to a straight-hinged veliger stage (Lovén, 1848; Lebour, 1938; Miyazaki, 1936; Franz, 1973; Ockelmann and Muus, 1978; Ó Foighil *et al.*, 1984). The complicated reproductive cycle of the North Eastern Atlantic species *Mysella bidentata* has been investigated in detail by Deroux (1961) and Ockelmann and Muus (1978). *M. bidentata* is one of the few bivalve species that produces dimorphic sperm. Sperm are stored in the suprabranchial chamber in three separate ways: in sac-like spermatophores, within a ventral fold in the floor of the gill chamber (termed the accessory male organ by Deroux), or attached in irregular masses to the abfrontal surfaces of the gill filaments. Ockelmann and Muus (1978) interpreted the accessory male organ as a seminal receptacle and argued that the production of spermatophores and dimorphic sperm indicated that outcrossing normally occurs in this species. The actual mechanism of sperm transfer is still unclear.

Mysella tumida (Carpenter, 1864) occurs in the North Eastern Pacific (Abbott, 1974) and relatively little is known about its reproductive cycle. It broods embryos in the gill chamber, is hermaphroditic, and its monomorphic sperm are atypical in that while in the testis they possess numerous microvilli that radiate from the middle piece (Ó Foighil, 1985b). This study aims to describe how sperm storage is achieved in *M. tumida* and to outline how sperm transfer may occur. The results presented may help explain how outcrossing and sperm storage are achieved by other ctenidial brooding bivalves.

Materials and Methods

Specimens of *Mysella tumida* were sampled intertidally at monthly intervals from August 1982 to September 1983 at Patricia Bay, Victoria, British Columbia, Canada. Thirty live individuals (>2.0 mm in valve length) per sample were dissected and examined with a dissecting microscope to determine the reproductive cycle. The incidence of developing embryos in the suprabranchial chamber and of sperm and eggs in the gonad were recorded. An additional thirty specimens were examined each month using light histology to investigate the incidence of sperm attachment to the gill filaments. Specimens were fixed in 2% glutaraldehyde (biological grade), decalcified in a 1:1 mixture of 2% ascorbic acid and 0.3 *M* NaCl (Dietrich and Fontaine, 1975) for 2–4 days, processed by routine methodology, and embedded in paraffin wax. Serial sections at 7 μm intervals were cut and then stained with Eriochrome cyanin (Chapman, 1977).

Gills and spermatophores were dissected from live specimens and fixed for 1 hour at 4°C in a 3:1 mixture of 4% glutaraldehyde and 1% osmium tetroxide in 3% NaCl

(Smith, 1983). They were then dehydrated in an acetone series, critical point dried, gold coated, and viewed with a JEOL JSM-35 scanning electron microscope. Gill filaments and spermatophores were fixed for transmission electron microscopy in 5% glutaraldehyde with 0.1 *M* sodium cacodylate buffer at pH 7.4, and 0.25 *M* sucrose, for one hour at room temperature. They were then rinsed in buffer solution and post-fixed in 1% osmium tetroxide in the same buffer for one hour at 4°C. Specimens were dehydrated in an ethanol series, embedded in Epon-812, and sectioned with glass knives on a Reichert ultramicrotome. Silver-grey sections were stained with uranyl acetate and lead citrate, and viewed with a Philips EM-300 transmission electron microscope.

The fertilization efficiencies of 50 individuals of *Mysella tumida* were investigated by dissecting out and examining broods of early embryos (4 cell–blastula stage). Embryonic development within each brood was synchronous and the proportion of unfertilized eggs (characterized by the absence of cleavage or polar bodies) was recorded.

Results

Reproductive cycle

The reproductive cycle of the Patricia Bay *Mysella tumida* population is outlined in Figure 1. Brooding individuals were detected between February and May 1983. There was a corresponding drop in the frequency of animals producing sperm during March and April, and in the number of individuals undergoing oogenesis from March to June. This pattern of seasonal reproduction is set against an environmental background which shows a marked seasonality in ambient temperature. From January to May 1983 some specimens were found to possess large masses of sperm cells attached to their gill filaments. The monthly flux in the frequency of animals with sperm on their gills broadly paralleled, but preceded by one month, the proportion of brooding individuals in the population.

Sperm-gill attachment

The *Mysella tumida* gill is composed of a pair of inner demibranchs, each formed by a descending and ascending lamella (Fig. 2). Both lamellae possess frontal and lateral cilia, and latero-frontal cirri, but differ in that the abfrontal surface of the ascending lamella is not ciliated (Fig. 3), whereas the descending lamella bears abfrontal cilia (Fig. 4). The ascending lamella is also the larger of the two. Sperm attachment occurs at the abfrontal surface of the constituent gill filaments in the ascending lamella. The attached sperm are typically aggregated to form distinct patches on the gill surface (Fig. 5). Within these patches, the gill filaments and ostia are obscured by the sperm flagella (Fig. 6) and the sperm heads are orientated with the acrosomal end facing the gill filaments (Fig. 7). The attached sperm cells are limited in their distribution to the abfrontal surface of the gill filament by the lateral cilia (Fig. 8).

Despite being orientated toward the gill filaments, the sperm heads are usually separated from the gill epithelium by a 2–8 μm gap. The gap between the sperm heads and the gill epithelium is spanned by fine thread-like processes (Fig. 9), which are extensions of the sperm plasmalemma where it lies closely apposed to the intact acrosomal vesicle (Fig. 10). These extensions are 30 nm in diameter and resemble the microvilli which radiate from the middle piece of *Mysella tumida* sperm while in the testis (Ó Foighil, 1985b). However, no microvilli are present on the middle piece of

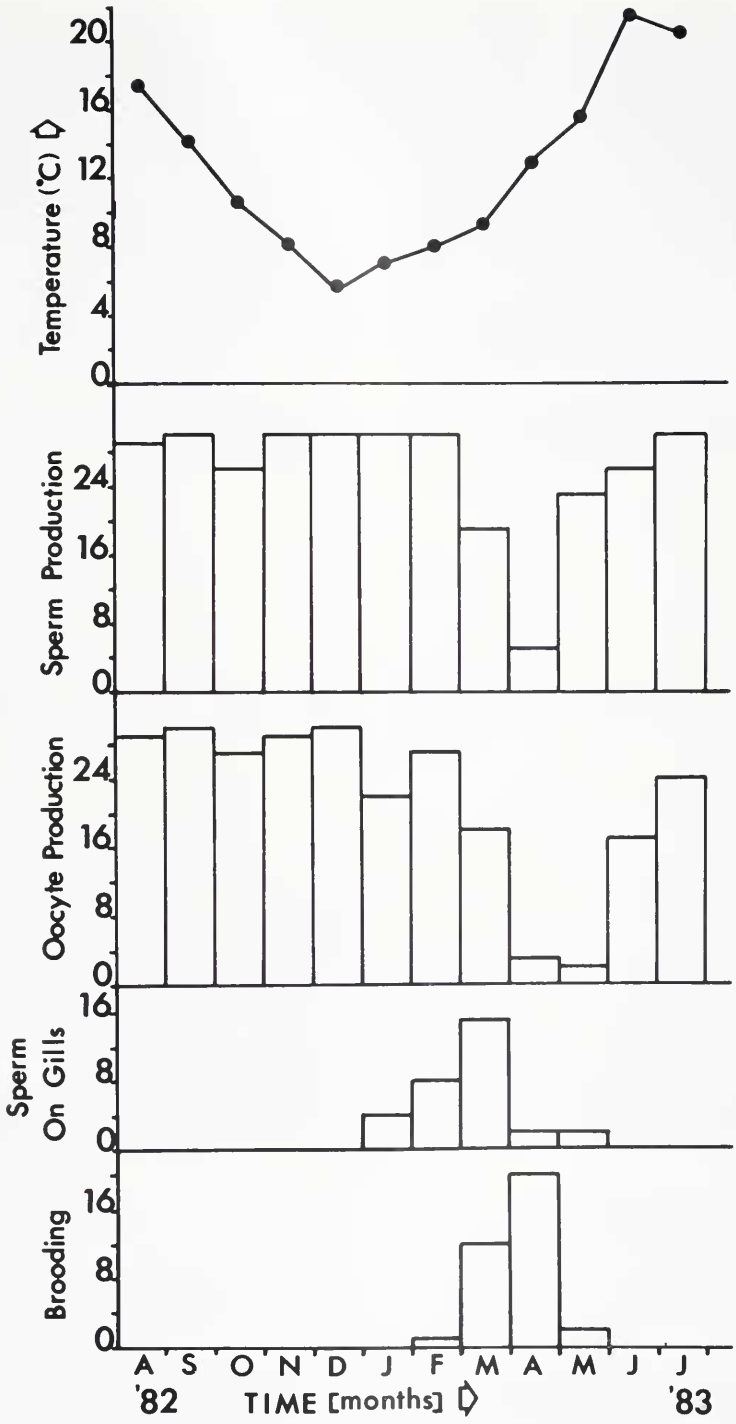

FIGURE 1. Outline of reproductive cycle of *Mysella tumida* at Patricia Bay from August 1982 to July 1983. Ambient surface water temperatures and the reproductive condition of 30 individuals per monthly sample is presented.

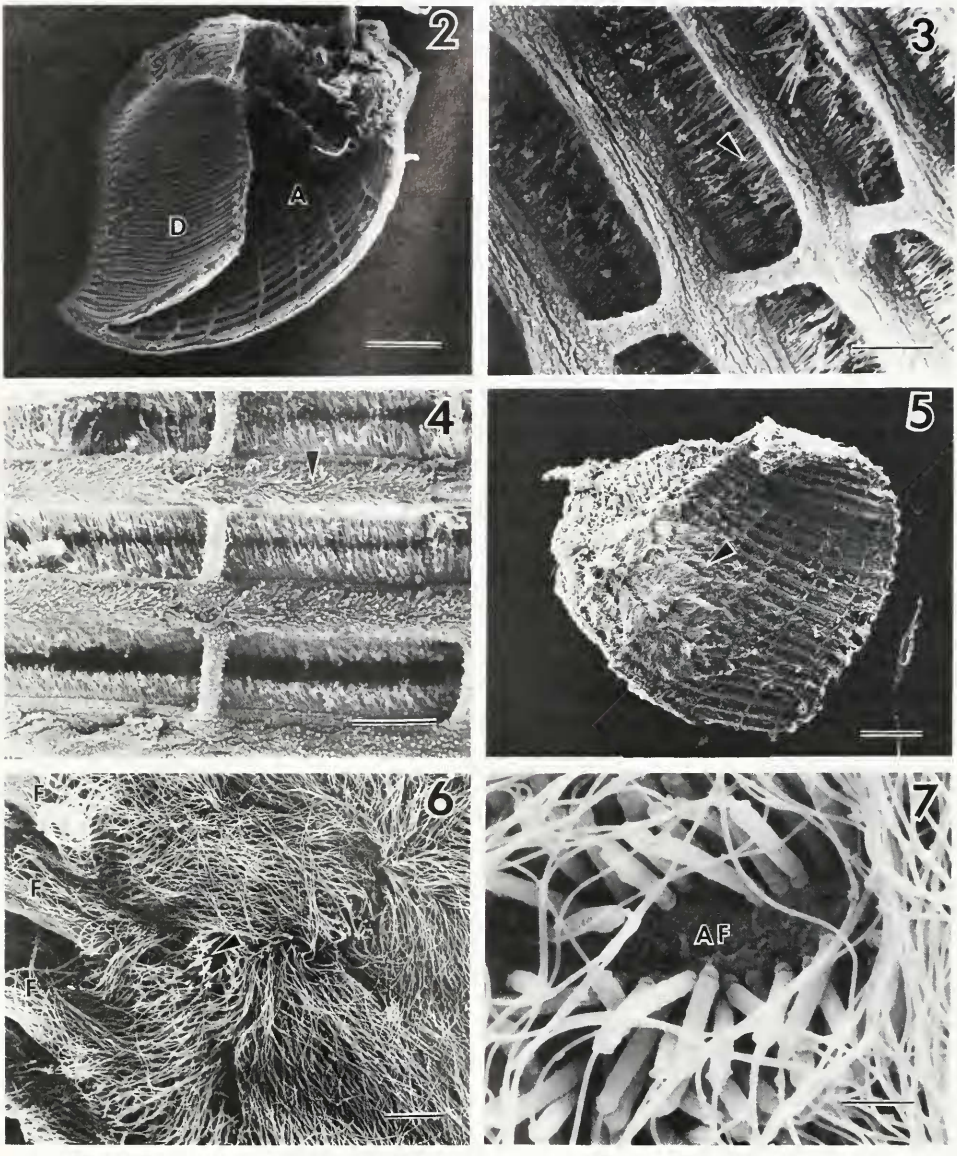

FIGURE 2. Scanning electron micrograph (S.E.M.) of *M. tumida* gill demibranch. A, ascending lamella; D, descending lamella. Scale = 100 μm.

FIGURE 3. S.E.M. of abfrontal surface of *M. tumida* ascending gill lamella. Note absence of abfrontal cilia. Arrow points to lateral cilia. Scale = 10 μm.

FIGURE 4. S.E.M. of abfrontal surface of *M. tumida* descending gill lamella. Arrow points to abfrontal cilia. Scale = 10 μm.

FIGURE 5. S.E.M. of *M. tumida* ascending gill lamella with attached sperm mass (see arrow). Scale = 100 μm.

FIGURE 6. S.E.M. of *M. tumida* sperm mass attached to gill filaments showing occlusion of ostia by sperm flagella. Arrow points to sperm heads. F, gill filaments. Scale = 30 μm.

FIGURE 7. S.E.M. of attached *M. tumida* sperm heads. Note orientation of acrosomal end toward abfrontal gill epithelium (AF). Scale = 5 μm.

M. tumida gill-attached sperm (Fig. 11). On reaching the abfrontal surface of the gill filaments, the acrosomal microvilli interdigitate with the gill filament microvilli (Fig. 12). The gill epithelium microvilli are shorter in length (<1.25 μm) and greater in diameter (up to 125 nm) than the acrosomal microvilli, so that both types may be readily distinguished. Membrane fusion between the two microvillar types was not observed, but they frequently come into close apposition (<14 nm) where their respective glycocalices make contact (Fig. 13).

Living gills bearing attached sperm were examined by light microscopy. The sperm masses were continually buffeted by powerful water currents generated by the lateral cilia and passed in through the gill ostia. It was not possible to determine if the constant flexing of these sperm cells was caused solely by the action of the lateral cilia. However, smaller masses of sperm attached to more sheltered portions of the gill chamber demonstrated a low level of flagellar movement by individual sperm cells. This indicates that, although tethered by microvilli, the attached sperm remain activated.

Eggs were fertilized by the stored spermatozoa after being spawned into the gill chamber. Brooding individuals were not observed to have sperm attached to their gill filaments. The fecundity of 50 individuals ranged from 221–1279 (number of eggs spawned) per individual. A total of 40 unfertilized eggs were detected among 39,660 developing embryos. This is equivalent to a mean fertilization efficiency of 99.9%.

Spermatophore production and transfer

Histological examination of animals collected to determine the reproductive cycle revealed spermatophores in the suprabranchial chambers of two individuals in February 1983. In both cases the spermatophores projected from the gonaduct opening into the gill chamber (Fig. 14). From January to March 1984 freshly collected *Mysella tumida* were held in finger bowls of seawater at 10°C and checked daily for evidence of sperm transfer. Fourteen individuals were observed releasing single spermatophores *via* their posterior exhalent siphons. They were elongate, delicate, transparent structures (Fig. 15), up to 0.6 mm in length, which floated freely in the finger bowls. Spermatophores contained masses of sperm cells (Fig. 16), many of which demonstrated a low level of flagellar activity, some spermatids and occasional oocyte fragments enclosed within a 0.2–0.6 μm thick wall. The spermatophore wall had no distinct substructure, and was composed of a layer of flocculant material with frequent electron translucent pockets (Fig. 17). No opening was discerned in the spermatophore wall. Some spermatophoric sperm had microvilli radiating from their acrosomal ends as well as from the middle piece (Fig. 18).

Newly released spermatophores were placed together with single specimens of *Mysella tumida* in finger bowls to determine if spermatophore uptake would occur. On contacting the extended foot of these highly mobile bivalves, the spermatophores were passed posteriorly by the cilia of the foot surface. Upon foot withdrawal, the spermatophores adhered to the shell surface, typically in the posterior-ventral region of the valves (Fig. 19). This was frequently (>20 occasions) observed in freshly collected individuals. In this position the spermatophores occlude the exhalent opening which leads into the suprabranchial chamber. Laboratory-held specimens retained the externally attached spermatophore for up to 4 days. During this time the innermost portion of the spermatophore wall that projected between the open valve margins was ruptured by occasional adductor muscle contractions. Sperm cells were observed being sucked into the suprabranchial chamber following sudden adductor muscle relaxations.

Spermatophores were not taken into the mantle cavity through the anterior in-

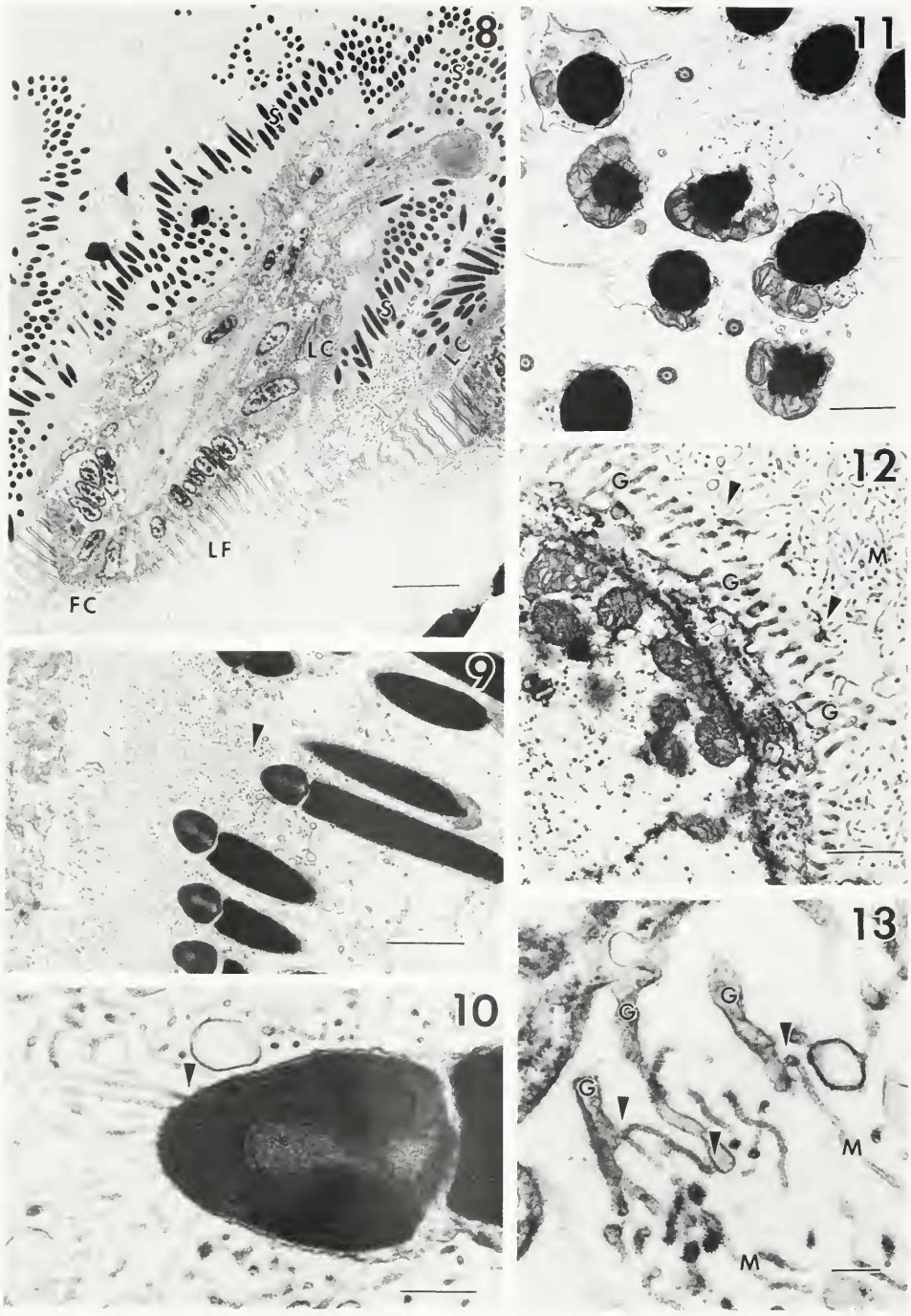

FIGURE 8. Transmission electron micrograph (T.E.M.) of cross-section through *M. tumida* gill filament with attached spermatozoa. FC, frontal cilia; LC, lateral cilia; LF, laterofrontal cirri; S, attached spermatozoa. Scale = 10 μm.

halent/pedal opening and sperm masses placed in the inhalent current did not pass to the suprabranchial chamber, but were transported to the mouth and ingested as described by Ockelmann and Muus (1978) for *Mysella bidentata.*

Discussion

Sperm storage and brooding are closely coordinated seasonal events in the reproductive cycle of *Mysella tumida.* The advent of sperm storage in the Patricia Bay population precedes by one month the onset of brooding. Brooding is also a seasonal phenomenon in *M. bidentata* populations (Ockelmann and Muus, 1978; Ó Foighil *et al.,* 1984), but individuals storing sperm are prevalent throughout most of the year (Ockelmann and Muus, 1978). The difference between the two species in the duration of sperm storage may reflect the more specialized sperm storage microenvironment found in *M. bidentata.* This involves a possible nutritive role for the oligopyrene sperm and the development of a distinct seminal receptacle (Ockelmann and Muus, 1978). *M. bidentata* sperm may also attach to the abfrontal surface of gill filaments, but the presence of two sites for sperm attachment in this species is of unknown significance.

While in the testis, *Mysella tumida* sperm microvilli are located on the middle piece (Ó Foighil, 1985b). After being packaged into spermatophores and released through the exhalent opening some sperm cells develop microvilli at the acrosomal end. Sperm cells dissected live from the gonad also develop acrosomally placed microvilli (pers. obs.). The relocation of the sperm microvilli may occur with sperm activation. It may be significant that in the two positions where the microvilli occur, the plasmalemma comes into close apposition (<14 nm) to the membranes of the underlying organelles, the acrosomal vesicle and mitochondria. Acrosomally placed microvilli offer some potential advantages over those located on the middle piece in achieving sperm attachment to the gill epithelium. In the former case, attachment cannot be disrupted by the undulating flagellum and a greater number of sperm cells may adhere per area of gill epithelium because the sperm are orientated with their long axis perpendicular and not parallel to the gill surface. The rod-like shape of the sperm head in *M. tumida* (Ó Foighil, 1985b) may also allow a more efficient packing of spermatozoa in spermatophores and on gill filament epithelia. *M. bidentata* eupyrene sperm heads are also rod-like in shape (Ockelmann and Muus, 1978).

Mysella tumida sperm are constantly buffeted by the water currents generated by the nearby lateral cilia, but remain firmly attached to the ascending gill filaments until the eggs are spawned into the suprabranchial chamber. Sperm-gill adhesion is perhaps achieved by glycoprotein crosslinking of the epithelial cell and sperm cell glycocalices. Similar forms of sperm storage have been discovered in a variety of invertebrate and

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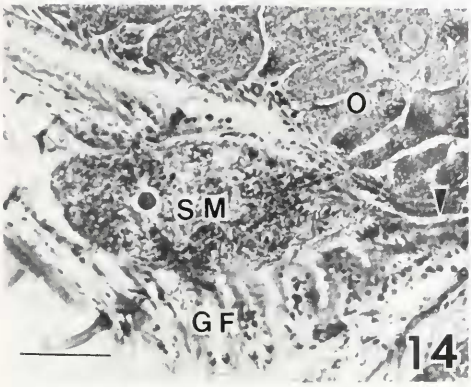
FIGURE 9. T.E.M. of longitudinal section through *M. tumida* sperm heads, revealing numerous microvilli (arrow) radiating from acrosomal region of spermatozoa toward gill epithelium. Scale = 2 μm.

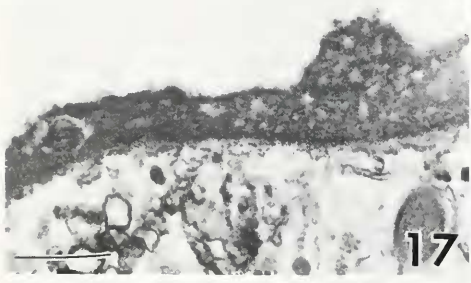
FIGURE 10. T.E.M. of median longitudinal section through *M. tumida* acrosomal vesicle. Sperm cell microvilli are apparent as extensions of the plasmalemma (arrow) where it comes into proximity with underlying acrosomal vesicle. Scale = 0.4 μm.

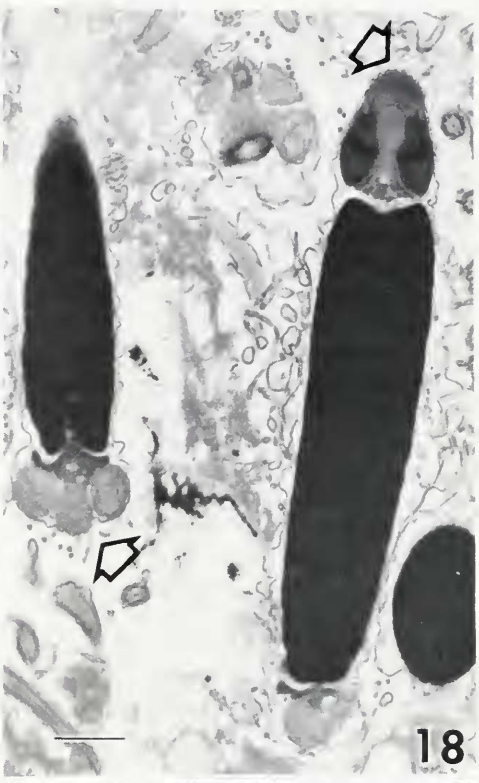
FIGURE 11. T.E.M. of section through middle pieces of attached *M. tumida* spermatozoa. Note absence of microvilli. Scale = 1 μm.

FIGURE 12. T.E.M. of section through *M. tumida* gill filament epithelial cell surface showing interdigitation (arrows) of gill filament microvilli (G) and spermatozoan microvilli (M). Scale = 1 μm.

FIGURE 13. T.E.M. of section through *M. tumida* gill filament epithelial cell surface revealing the close apposition of gill filament microvilli (G) and spermatozoan microvilli (M). Arrows indicate areas of close contact between both microvillar types. Scale = 0.2 μm.







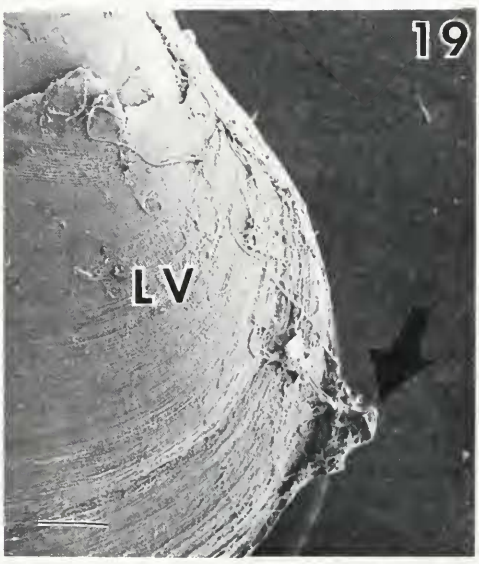


FIGURE 14. Light micrograph (L.M.) of longitudinal section through *M. tumida* spermatophore, projecting from the gonoduct (arrow) into suprabranchial chamber. GF, gill filaments; O, developing oocytes in gonad; SM, spermatophore. Scale = 100 μm.

FIGURE 15. L.M. of newly released *M. tumida* spermatophore. Scale = 80 μm.

FIGURE 16. L.M. of cross-section through *M. tumida* spermatophore. Note numerous spermatozoa enclosed by thin spermatophore wall. Scale = 40 μm.

vertebrate species *e.g.*, the polychaete *Spirorbis spirorbis* (Daly and Golding, 1977; Picard, 1980), the gastropod *Cochlostoma montanum* (Giusti and Selmi, 1985), the reptile *Thamnophis sirtalis* (Hoffman and Wimsatt, 1972), and a variety of bat species (Racey and Potts, 1970; Racey, 1979; Mori *et al.*, 1982; Andrucetti *et al.*, 1984).

Spermatophore production in the Bivalvia is known to occur in a small number of galeommatacean species (Ó Foighil, 1985a), which includes *Mysella bidentata* (Deroux, 1961; Ockelmann and Muus, 1978). Deroux (1961) concluded that *M. bidentata* spermatophores are formed by the specialized sperm-storing portion of the gill that he termed the accessory male organ. Ockelmann and Muus (1978) suggest that due to the elongate shape of the spermatophores and their typical position opposite the genital pore in the suprabranchial chamber, that in this species the spermatophores are formed in the gonadal duct. This interpretation is supported by observations on *M. tumida* where there is no accessory male organ and spermatophores have been observed protruding from the gonadal ducts. It is probable that the spermatophore wall is secreted by the gonadal duct epithelium and is moulded around the emerging sperm mass. In the polychaete species *Polydora ligni* and *P. websteri*, spermatophores are formed in the nephridia where sperm masses are surrounded by a layer of adherent microvilli shed from the nephridial epithelium (Rice, 1980). The spermatophore wall of *M. tumida* differs in that it is composed of amorphous material, which would indicate an alternate mode of construction.

Spermatophore production in the Galeommatacea occurs in species which are normally aggregated, usually on or around a host animal (Ó Foighil, 1985a). *Mysella tumida* has been previously found in the oxygenated sediment zone surrounding burrows of the holothurian *Leptosynapta clarki* (Ó Foighil and Gibson, 1984). In Patricia Bay *M. tumida* are typically clustered around the ends of the tubes of the polychaete *Mesochaetopterus taylori*. Up to 120 individuals may occupy a small volume of sediment around the tube of an individual *Mesochaetopterus* (pers. obs.).

The aggregated nature of the *M. tumida* populations provides feasible conditions for sperm transfer to occur *via* spermatophores. Laboratory observations on newly sampled specimens indicate that spermatophore release through the exhalent opening occurred in the Patricia Bay population from January to April in 1984. Although not all the details of sperm transfer in this species are yet obvious, it is clear that once released from the suprabranchial chamber, sperm can re-enter the gill chamber only through the exhalent opening. *M. tumida* is an indirect deposit feeder (pers. obs.) and uses its foot during the feeding process in a manner similar to *M. bidentata* (Ockelmann and Muus, 1978). Spermatophores contacting the foot become attached to the posterior-ventral shell margin and occlude the exhalent opening. Adductor muscle contractions lead to the rupturing of the spermatophore wall and the inhalation of sperm, but subsequent events involving the attachment of the spermatozoa to the gill filaments were not observed. For this to occur, some behavioral modification of the normal suprabranchial water flow, most likely a reduction in the activity of the lateral cilia, is necessary.

Spermatophores, temporary dwarf males and complemental males are utilized as methods of bulk sperm transfer in the Galeommatacea (Ó Foighil, 1985a). The specialized mode of sperm transfer in *Mysella bidentata* involves the formation of di-

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FIGURE 17. T.E.M. of section through spermatophore wall. Scale = 0.4 μm.

FIGURE 18. T.E.M. of longitudinal section through *M. tumida* spermatophoric sperm cells. Microvilli are present at both the acrosomal end and the middle piece (arrows). Scale = 0.9 μm.

FIGURE 19. S.E.M. of posterior-ventral region of *M. tumida* valves. Note spermatophore (arrow) lodged between valve margins. LV, left valve. Scale = 100 μm.

morphic sperm, spermatophores, and seminal receptacles and may result in enhanced fertilization success (Ockelmann and Muus, 1978). *M. tumida* achieves a high degree of fertilization efficiency (99.9%) by employing some of these specializations; *e.g.* bulk sperm transfer of spermatozoa followed by sperm storage at the fertilization site until the eggs are spawned. It seems likely that other Galeommatacean species that undergo bulk sperm transfer benefit from a similarly high fertilization success. Recent *in situ* work on spawning in echinoids indicates that in broadcast spawners, zygote production could be much less than egg production, unless the aggregation of synchronous spawners counter-acts excessive sperm dilution (Pennington, 1984). *M. tumida* produces a small amount of sperm relative to broadcast spawners, but may avoid the reproductive hazards of excessive sperm dilution by fertilizing the eggs in a correspondingly small water body—the suprabranchial chamber.

Localized fertilization in or on the parent animal is an obvious prerequisite for the brooding habit and bulk sperm transfer is an effective method of achieving localized fertilization. Therefore, the development of bulk sperm transfer methods may have preceded the brooding habit in many outcrossing invertebrates. In bivalves these methods include pseudocopulation (Townsley *et al.*, 1965) and sperm ball production (Coe, 1931; Andrews, 1979) as well as the aforementioned reproductive specializations. A possible advantage for localized fertilization is that it results in a greater degree of fertilization efficiency, which is proportionally more important for animals of low fecundity. An alternate method of achieving localized fertilization is through selfing, which has been reported from a variety of bivalve species (Thomas, 1959; Castanga and Duggan, 1971; Chanley and Chanley, 1980; Morton, 1980; Kraemer, 1983). There is indirect evidence that *Mysella tumida* normally outcrosses: (1) the almost equal male and female investment in the gonad (Ó Foighil, 1985b) which is indicative of outcrossing (Heath, 1979); (2) the production and release of spermatophores; and (3) the occurrence of sperm storage even though this species is a simultaneous hermaphrodite. The possibility of facultative selfing in *M. tumida* as reported from *Corbicula fluminea* (Kraemer, 1983), however, is not ruled out.

ACKNOWLEDGMENTS

My thanks to Dr. A. R. Fontaine, Louise Bickell-Page, Allan Gibson, and Richard Gustafson for critically reviewing early drafts. The constructive comments of two anonymous reviewers helped in shaping this publication. The work was supported by a University of Victoria Graduate Fellowship to the author and an NSERC operating grant to Dr. A. R. Fontaine.

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